Journal of Thermal Analysis and Calorimetry, Vol. 95 (2009) 3, 721–725

EFFECT OF PHALLOIDIN ON FILAMENTS POLYMERIZED FROM HEART MUSCLE ADP-ACTIN MONOMERS

Andrea Vig, Réka Dudás, Tünde Kupi, J. Orbán, G. Hild, D. Lőrinczy and M. Nyitrai[*]

University of Pécs, Faculty of Medicine, Department of Biophysics, Szigeti str. 12, Pécs 7624, Hungary

The effect of phalloidin on filaments polymerized from ADP-actin monomers of the heart muscle was investigated with differential scanning calorimetry. Heart muscle contains α-skeletal and α-cardiac actin isoforms. In the absence of phalloidin the melting temperature was 55°C for the α-cardiac actin isoform and 58°C for the α-skeletal one when the filaments were generated from ADP-actin monomers. After the binding of phalloidin the melting temperature was isoform independent (85.5°C). We concluded that phalloidin stabilized the actin filaments of α-skeletal and α-cardiac actin isoforms to the same extent when they were polymerized from ADP-actin monomers.

Keywords: *calorimetry, cardiac ADP-F-actin, isoform, phalloidin, thermodynamics*

Introduction

Actin is one of the most abundant proteins in biological systems and has many important functions in cells [1–4]. Two isoforms can be found in the muscle tissues; the α-cardiac and the α-skeletal isoforms. The α-cardiac actin isoform differs from α-skeletal isoform by three amino acids, which is considered to be a small difference relative to the total number of amino acids (375). Skeletal muscle contains only skeletal actin isoform, but in cardiac muscle both isoforms are expressed. The contribution of the skeletal actin isoform in cardiac muscle is 16–24% of the total actin content [5, 6].

Actin can exist in its monomeric (G-actin) and filamentous (F-actin) form in cells [7]. The monomer actin binds an ATP and a divalent magnesium ion under physiological circumstances. The quality of the bound divalent cation affects the molecular properties of the actin [8–12]. Actin monomers polymerize to the filamentous form. The polymerization is accompanied by the hydrolysis of the bound ATP. ATP is not essential for filament assembly, therefore ADP-actin monomers can also form filaments [13, 14]. Janmey *et al*. suggested that the energy released by the hydrolysis of ATP could be stored in the protein and affects the filament structure [15]. The inter-monomer flexibility of filaments assembled from skeletal ADP-actin monomers was found to be greater than the one assembled from ATP-bound monomers [13].

Phalloidin, a cyclic peptide from *Amanita phalloides* can tightly bind to the actin filaments and stabilizes their structure [16, 17]. Phalloidin stabilized actin filaments are extensively used for in vitro studies [18, 19]. Visegrády *et al*. showed that phalloidin made the ATP-actin more rigid, and that the binding of one phalloidin can have an effect on the conformation of seven actin protomers [20, 21].

Filaments polymerized from ADP-actin or ATP-actin monomers can be studied with differential scanning calorimetry (DSC). Calorimetry is a powerful method to determine the thermodynamic properties of proteins and to study differences between them on the molecular level. DSC is extensively used to characterize the thermodynamic properties of actin filaments [20–25]. Previous studies showed that filaments polymerized from ATP monomers were thermodynamically more stable than the ones polymerized from ADP-monomers, independently of their skeletal or cardiac origin. Actin filaments polymerized from ADP-monomers of cardiac muscle showed two transition peaks characterized with different T_m values, indicating that the denaturation temperature for the two actin isoforms (cardiac, skeletal) was different [22].

The aim of our study was to investigate the effect of phalloidin on the thermal properties of actin filaments from ADP-actin monomers of the cardiac tissue with DSC [26–28]. Based on the observations we concluded that the isoform specific differences between the thermal stability of the actin filaments polymerized from ADP-actin monomers disappeared after the binding of phalloidin. Phalloidin-binding stabilized the structure of these filaments, and proved to be cooperative inducing long-range allosteric interactions along the actin filaments.

* Author for correspondence: miklos.nyitrai@aok.pte.hu

1388–6150/$20.00

Akadémiai Kiadó, Budapest, Hungary
Springer, Dordrecht, The Netherlands

Experimental

Materials and methods

Chemicals

KCl, $MgCl_2$, $CaCl_2$, MOPS, hexokinase, *D*-glucose, EGTA and phalloidin were purchased from Sigma-Aldrich (Budapest, Hungary). ATP, ADP and β-mercaptoethanol were obtained from Merck (Darmstadt, Germany). NaN_3 was purchased from Fluka (Lausanne, Switzerland).

Actin preparation

Cardiac actin was prepared from acetone powder of bovine heart muscle [29, 30]. The calcium saturated actin monomers were stored in 2 mM MOPS buffer (pH 8.0) containing 0.2 mM ATP, 0.1 mM $CaCl_2$, 0.1 mM β-mercaptoethanol and 0.005% NaN_3 at 4°C (Spudich and Watt, 1971). Actin concentration was determined by using the absorption coefficient 0.63 mL mg^{-1} cm^{-1} at 290 nm [31].

Preparation of ADP-bound actin filaments

The bound calcium (Ca^{2+}) in the actin monomers was changed for magnesium by incubating the samples for 5 min at room temperature in the presence of 0.2 mM EGTA and 0.1 mM $MgCl_2$. The nucleotide exchange on the filaments was done by incubating the actin for 1 h at 4°C in the presence of 1.65 mg mL^{-1} hexokinase, 0.5 mg mL^{-1} glucose and 1 mM ADP [32]. When ATP spontaneously dissociated from the actin monomers, the hexokinase hydrolyzed the free ATP and phosphorylated the glucose depleting the ATP content of the buffer. The ADP then bound to the nucleotide-binding cleft of the actin.

Preparation of phalloidin bound actin filaments

To create phalloidin bound F-actin we added phalloidin at the same time as the polymerization was started. Since cardiac-actin polymerize slower than skeletal-actin, and ADP-bound actin, polymerize even slower, the polymerizing samples were incubated for 8 h at room temperature to ensure complete polymerization.

DSC measurements

The calorimetric measurements were performed with a Setaram Micro DSC-II calorimeter. The DSC measurements were carried out in the temperature range of 0–100°C. The heating rate was 0.3 K min^{-1}. In each case the samples were heated twice to check the fully irreversible denaturation of the protein. The polymerization buffer lacking actin and phalloidin was the reference solution during the DSC measurements. The actin concentration was 69 μM (3 mg mL^{-1}). All the DSC data were analyzed with Microcal Origin Software (version 7.5).

Results and discussion

In this study our aim was to describe the phalloidin-induced conformational changes on actin filaments polymerized from ADP-actin monomers of the heart muscle. To achieve this aim we used the method of differential scanning calorimetry. Previously this method proved to be able to distinguish between the different actin isoforms (α-skeletal and α-cardiac components) of heart muscle actin provided that the filaments were generated from ADP-actin monomers [22]. In the present study the calorimetric experiments were carried out on actin filaments polymerized from ADP-actin monomers in the presence of 2 mM $MgCl_2$ and 100 mM KCl in the absence and presence of phalloidin. The sample and the appropriate reference solution were heated in the range of 0 to 100°C under isobaric conditions as described in the Materials and Methods section. The difference between the energy input of the instrument to the sample and the reference cell was recorded as the function of temperature.

The obtained transition curves can be characterized by the melting temperature (T_m) where the transition curve reaches its maximal amplitude. The T_m value gives information about the thermodynamic stability of the different forms of proteins. The higher T_m value correlates with a thermodynamically more stable protein conformation.

Cardiac muscle contains both α-cardiac isoform of the actin molecule and α-skeletal actin isoform. Previous observations showed that the transition curve of cardiac muscle filaments show two melting temperatures belonging to the two isoforms of the actin [22]. Considering the T_m values it is possible to compare the thermodynamic stability of the different isoforms [33]. The lower T_m observed for α-cardiac actin filaments indicated that the structure of the isoform was thermodynamically less stable than the α-skeletal part of the actin population [22].

We performed our measurements to determine the melting temperatures of the cardiac ADP-actin filaments in the presence of phalloidin. Previous work showed that α-skeletal ADP-actin filaments have a higher melting temperature than α-cardiac ADP-actin filaments. The value of T_m was 54±2°C for the cardiac component, and 58±2°C for the skeletal component, respectively [22]. Previous studies also revealed that phalloidin stabilizes the skeletal ATP-actin filaments shifting the melting temperature from 64.1 to 82.3°C [34]. In our experiments the DSC curve of ADP-actin filaments with-

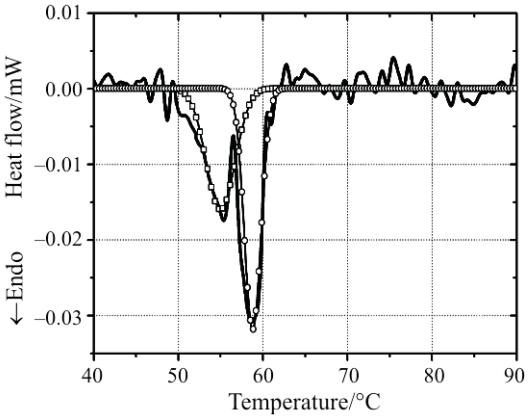

Fig. 1 The thermal denaturation curve of cardiac tissue origin F-actin without phalloidin. The peaks represent the Gaussian fits according to separate □ – α-cardiac and ○ – α-skeletal population of the ADP-bound actin filament. The T_m values were 54.7 and 58.8°C for the filaments polymerized from ADP-actin monomers of the cardiac and skeletal actin isoforms

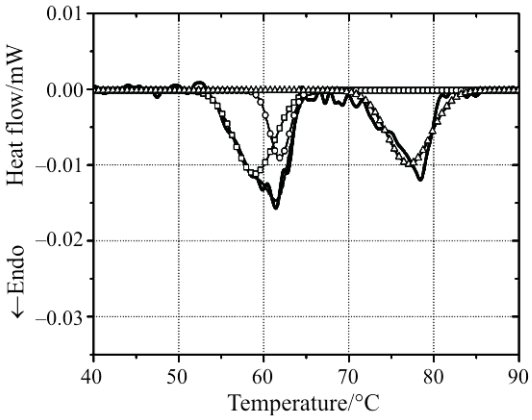

Fig. 2 The thermal denaturation curve in the case of 1:0.2 actin:phalloidin ratio. The experimentally obtained curve was decomposed to three Gaussians. The Gaussian curves corresponding to the phalloidin bound actin filaments with △ (77.1°C) and to the cardiac component with □ (58.9°C) and skeletal isoform component with ○ (61.9°C) of the unaffected filaments are indicated

out phalloidin showed two separated peaks, which were attributed to the two actin isoforms (Fig. 1). The melting temperatures for the cardiac and skeletal isoform population were 55.2±0.4 and 58.8±0.4°C, respectively. Double Gaussian fit was made to the heat transition curves. This approach was successfully used in the previous works [22]. The melting temperatures resolved by the Gaussian fit were 54.7±0.2 and 58.8±0.6°C for the cardiac and skeletal isoforms, respectively. These T_m values were close to the obtained peak values from the original curves.

When the phalloidin was added to the molar ratio of 1:0.2 (actin:phalloidin) two major transition peaks appeared. The first peak was at 61.4±0.5 and the second at 78.4±0.5°C (Fig. 2). The first peak is related to the protomers which were unaffected by the binding of phalloidin. This heat transition curve was the combination of the contributions from the two actin isoforms. This first transition could be decomposed into two peaks corresponding to the cardiac and skeletal isoform by using Gaussian fits. The decomposition resulted in melting temperatures of 58.9 and 61.87°C. The second major peak represented the actin filaments which were affected by the binding of phalloidin. The melting temperature corresponding to this transition was higher (78.4°C) than the ones characterizing either of the unaffected actin isoforms (Fig. 2). These observation showed that the phalloidin binding stabilized both of the actin isoforms, and the differences between the isoforms observed without the effect of phalloidin disappeared after the phalloidin stabilized the filaments.

When the phalloidin concentration was increased – but still kept under substoichiometric condition – to 1:0.5 actin:phalloidin concentration ratio the recorded data showed one transition curve with the melting temperature of 80.1°C. At stoichiometric phalloidin concentration (1:1) the melting temperature of the actin filaments shifted to an even higher value, to 85.5°C. The observation that the T_m value was lower at 1:0.5 actin:phalloidin than at 1:1 actin:phalloidin concentration was probably due to the fusion of the unseparated peaks belonging to the phalloidin bound and unbound protomers. At 1:1 actin:phalloidin concentration ratio the transition peak referring to the actin filaments not affected by the toxin completely disappeared from the heat transition curve and only the shifted transition peak of the phalloidin-affected filaments appeared (Fig. 3).

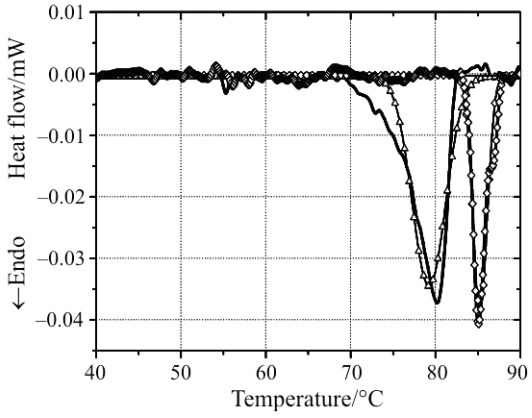

Fig. 3 The recorded curves in case of △ – 1:0.5 and ◇ – 1:1.1 actin:phalloidin concentration ratios. These transition curves showed single transition peak in the two cases with T_m values of 80.1 and 85.5°C, respectively

These data showed that the binding of phalloidin to actin filaments polymerized from ADP-actin monomers of the heart muscle resulted in only one transition peak with a single melting temperature at around 85°C and abolished the separation between the melting temperatures of the actin isoforms.

Previous measurements showed that the binding of phalloidin is propagated to distant protomers from the binding site of the drug [21]. In the lack of cooperativity one would expect that half of the actin filaments were unaffected by the binding of phalloidin at the phalloidin:actin ratio of 0.5:1. In our experiments the contribution of the unaffected actin population was much smaller, indicating that the bound phalloidin could influence the conformation of more than one actin protomer. We concluded that the small contribution of the actin filaments unaffected by the phalloidin to the heat transition peak at 1:0.5 actin:phalloidin ratio indicated that the binding of phalloidin to the filaments from ADP-actin monomers of the cardiac muscle was cooperative.

Conclusions

In the present study we described the thermal stability of actin filaments polymerized from cardiac ADP-actin monomers to characterize the phalloidin binding induced structural changes in the protein. The denaturation curves of filaments from ADP-actin monomers showed two thermal transition peaks. The two peaks were attributed to the two actin isoforms – α-cardiac and α-skeletal – characteristic for the heart muscle. This observation is in good agreement with previous works, when in the case of cardiac actin filaments built up from ADP-actin monomers showed the same thermal transitions and melting temperatures. We applied phalloidin to examine its effect on the thermal stability of the actin filaments from heart muscle and found that the melting temperature increased for the actin filaments after the binding of phalloidin. This observation indicated that phalloidin had a stabilizing effect on the filaments. The calorimetric data also revealed that binding of phalloidin to the actin filament abolished the separation between the two transition curves observed for the two actin isoforms in the absence of the drug. Our conclusion is that phalloidin can stabilize the ADP-actin filaments as well and its strong influence on the actin filaments can vanish the difference between actin filaments polymerized from cardiac and skeletal ADP-actin monomers. When the phalloidin was applied in substoichiometric concentration – at a 1:0.5 actin:phalloidin concentration ratio the stabilizing effect by the drug was already apparent. These results also showed that the binding of phalloidin to the cardiac ADP-actin filaments was cooperative.

Acknowledgements

This work was supported by grants from the Hungarian Scientific Research Fund (GVOP grant No. 3.2.1.-2004-04-0190/3.0 (Gábor Hild)). The Setaram Micro DSC-II was purchased with a grant (CO-272 (Dénes Lőrinczy)) from the Hungarian Scientific Research Fund. Miklós Nyitrai holds a Wellcome Trust International Senior Research Fellowship in Biomedical Sciences.

References

1 H. H. Chowdhury, M. R. Popoff and R. Zorec, Pflugers Arch., 439 (2000) R148.
2 P. Cossart, Curr. Opin. Cell Biol., 7 (1995) 94.
3 C. Lamaze, L. M. Fujimoto, H. L. Yin and S. L. Schmid, J. Biol. Chem., 272 (1997) 20332.
4 R. J. Pelham and F. Chang, Nature, 419 (2002) 82.
5 H. R. Bergen 3rd, K. Ajtai, T. P. Burghardt, A. I. Nepomuceno and D. C. Muddiman, Rapid Commun. Mass Spectrom., 17 (2003) 1467.
6 J. Vandekerckhove, G. Bugaisky and M. Buckingham, J. Biol. Chem., 261 (1986) 1838.
7 J. E. Estes, L. A. Selden, H. J. Kinosian and L. C. Gershman, J. Muscle Res. Cell Motil., 13 (1992) 272.
8 G. Hild, M. Nyitrai, J. Belágyi and B. Somogyi, Biophys. J., 75 (1998) 3015.
9 M. Nyitrai, G. Hild, J. Belágyi and B. Somogyi, Biophys. J., 73 (1997) 2023.
10 M. Nyitrai, G. Hild, J. Belágyi and B. Somogyi, J. Biol. Chem., 274 (1999) 12996.
11 G. Hild, M. Nyitrai and B. Somogyi, Eur. J. Biochem., 269 (2002) 842.
12 M. Nyitrai, G. Hild, Z. Lakos and B. Somogyi, Biophys. J., 74 (1998) 2474.
13 M. Nyitrai, G. Hild, N. Hartvig, J. Belágyi and B. Somogyi, J. Biol. Chem., 275 (2000) 41143.
14 B. Gaszner, M. Nyitrai, N. Hartvig, T. Kőszegi, B. Somogyi and J. Belágyi, Biochemistry, 38 (1999) 12885.
15 P. A. Janmey, S. Hvidt, G. F. Oster, J. Lamb, T. P. Stossel and J. H. Hartwig, Nature, 347 (1990) 95.
16 H. Faulstich, A. J. Schafer and M. Weckauf, Hoppe Seylers Z. Physiol. Chem., 358 (1977) 181.
17 Y. Miyamoto, M. Kuroda, E. Munekata and T. Masaki, J. Biochem., 100 (1986) 1677.
18 M. R. Bubb, I. Spector, B. B. Beyer and K. M. Fosen, J. Biol. Chem., 275 (2000) 5163.
19 M. R. Bubb, A. M. Senderowicz, E. A. Sausville, K. L. Duncan and E. D. Korn, J. Biol. Chem., 269 (1994) 14869.
20 B. Visegrády, D. Lőrinczy, G. Hild, B. Somogyi and M. Nyitrai, FEBS Lett., 565 (2004) 163.
21 B. Visegrády, D. Lőrinczy, G. Hild, B. Somogyi and M. Nyitrai, FEBS Lett., 579 (2005) 6.
22 J. Orbán, D. Lőrinczy, M. Nyitrai and G. Hild, Biochem. Biophys. Res. Commun., 368 (2008) 696.
23 D. Lőrinczy and J. Belágyi, J. Therm. Anal. Cal., 90 (2007) 611.
24 D. Lőrinczy, Zs. Vértes, F. Könczöl and J. Belágyi, J. Therm. Anal. Cal., 95 (2009) 721.
25 R. Dudás, T. Kupi, A. Vig, J. Orbán and D. Lőrinczy, J. Therm. Anal. Cal., 95 (2009) 709.

26 D. I. Levitsky, M. A. Ponomarev, M. A. Geeves, V. L. Shnyrov and D. J. Manstein, Eur. J. Biochem., 251 (1998) 275.
27 A. Muhlrad, I. Ringel, D. Pavlov, Y. M. Peyser and E. Reisler, Biophys. J., 91 (2006) 4490.
28 D. Lőrinczy, F. Könczöl, B. Gaszner and J. Belágyi, Thermochim. Acta, 322 (1998) 95.
29 G. Feuer, F. Molnár, E. Pettkó and F. B. Straub, Hung. Acta Physiol., 1 (1948) 150.
30 J. A. Spudich and S. Watt, J. Biol. Chem., 246 (1971) 4866.
31 T. W. Houk, Jr. and K. Ue, Anal. Biochem., 62 (1974) 66.
32 G. Drewes and H. Faulstich, J. Biol. Chem., 266 (1991) 5508.
33 D. M. L. Ladbury, J. Biocalorimetry 2: Applications of Calorimetry in the Biological Sciences. Ltd. JWS, Editor 2004.
34 J. Orbán, D. Lőrinczy, G. Hild and M. Nyitrai, Biochemistry, 47 (2008) 4530.

DOI: 10.1007/s10973-008-9404-5